Immunoaffinity Chromatography in the Detection of Dexamethasone in Equine Urine

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Abstract

Due to the widespread use of dexamethasone in racing horses, mostly in low doses by intra-articular administration for the treatment of inflammatory processes, a method is developed to detect this drug in horse urine samples using liquid–liquid extraction followed by immunoaffinity chromatography. Liquid chromatography with diode-array detection is used for the identification of the drug. The use of immunoaffinity columns enhances the selectivity of the analysis, and the results show that dexamethasone can be detected up to 28 h after intra-articular administration.

Introduction

Corticosteroids are drugs used in veterinary medicine, mostly for the treatment of inflammation. Their anti-inflammatory effects occur quite independent of the type of agent that elicits the response. The remarkable reduction in the inflammatory process and consequent supression of pain enables the corticosteroids to be widely used for the treatment of musculoskeletal diseases in athletic horses (1). Dexamethasone (DEX) is a synthetic corticosteroid often administered to horses intramuscularly or intra-articularly at doses ranging from 2.5 to 25 mg per total body weight. These low doses and the intense biotransformation of DEX (2,3) allied with its inherent chemical characteristics bring serious difficulties in detecting this drug.

It is recommended by the Accreditation Requirements and Operating Criteria for Horseracing Laboratories (International Laboratory Accredation Cooperation [ILAC], 1994) that mass spectrometry or a similar definitive technique, if applicable to the analyte in question, must be used for the identification of prohibited substances (4). The ideal technique for the chemical identification of DEX in urine samples is liquid chromatography coupled with mass spectrometry (LC–MS). However, due to the cost of this equipment, it is not available to all laboratories. In this case, a good option is the use of

immunoaffinity chromatography (IAC) prior to the identification procedure. The specificity of immunoaffinity chromatography results in highly purified urine extracts, a characteristic that enhances the specificity and selectivity of the final identification.

This paper describes a method for the detection of DEX in racehorse urine samples using IAC after liquid–liquid extraction (LLE) followed by liquid chromatography with diode-array detection (LC–DAD).

Experimental

Chemicals and materials

DEX was obtained from Frumtost S/A (São Paulo, Brazil). Ethanol, dichloromethane, ammonium chloride, and ammonium hydroxide were analytical reagent grade (Merck, Rio de Janeiro, Brazil). Methanol and acetonitrile were HPLC grade (EM Science, Gibbstown, NJ). The water used was freshly distilled, deionized, and purified in Milli Q plus equipment (Millipore, Bedford, MA). Immunoaffinity columns were supplied by Randox Laboratories (Crumlin, Antrim, UK). Dexadreson Forte injectable solution (1.31 mg/mL DEX 21-sodium phosphate and 2.66 mg/mL DEX 21-β-phenylpropionate) was obtained from Venie Veterinär Chemie (Germany). The ammonium buffer was pH 9.2 (100 mL NH_4OH added to a solution of 200 g NH_4Cl in 500 mL water).

Preparation of standards

A standard solution of DEX (1 mg/mL) was prepared in methanol. Working solutions of 100, 10, and 1 µg/mL were also prepared in methanol.

Administration procedure

A five-year-old thoroughbred mare weighing 450 kg received an intra-articular dose of 6 mg of Dexadreson Forte. Naturally voided urine samples were collected from 2–56 h after administration and stored at 4°C until the time of analysis.

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Sample treatment

Aliquots (60 mL) of urine samples were alkalinized with 6 mL of ammonium buffer and extracted with 60 mL of dichloromethane–ethanol (95:5, v/v). After agitation (3 min) and centrifugation (900 g, 10 min), the organic layer was filtered through qualitative filter paper and evaporated to dryness at 40°C under a nitrogen flow. The residues were dissolved in 8 mL of the wash solution supplied with the immunoaffinity columns kit followed by the steps recommended by the supplier's protocol. The final residues were resuspended in 50 μL of the mobile phase and analyzed by LC–DAD.

LC–DAD conditions

A Hewlett-Packard (Palo Alto, CA) 1090M liquid chromatograph equipped with a DAD and an ODS-Hypersyl column (100 × 4.6 mm, 5 μm particle size) was used in the experiment. The water–acetonitrile mobile phase (70:30, v/v) was isocratic and had a flow rate of 1.0 mL/min. A wavelength of 254 nm was used, and spectral acquisition was in the range of 220–400 nm. The injected volume was 25 μL.

Detection limit, recovery, and precision

The calibration curve for quantitation of DEX was prepared by repeatedly (six replicates) injecting standard solutions corresponding to the following amounts of the drug: 25, 50, 100, 250, and 500 ng.

For the detection limit, aliquots (60 mL) of horse urine sample were spiked with 200 and 250 ng of DEX (six replicates) and processed as described above. The detection limit was determined by comparing the ultraviolet (UV) spectra of the spiked sample and the standard, adopting a match quality greater than 95%. For the recovery study, six aliquots (60 mL) of horse urine sample were spiked with 250 ng and processed as described above. The quantitation of DEX was determined by using the calibration curve, and the recovery was calculated from these values. The recovery was determined after the first, fifth, and tenth use of the same immunoaffinity column.

The precision (CV [%]) was obtained from the same samples used in the recovery study. The intraday precision was obtained from six replicates in the same day, and the interday precision was obtained from three replicates in two consecutive days.

Results and Discussion

Due to the very low concentration of DEX in horse urine, it is necessary to use a sensitive, low-background method for its detection in biological matrices. Preliminary studies for the extraction of DEX from horse urine samples using either LLE or immunoaffinity columns showed insufficient sensitivity for the detection of this drug when administered intra-articularly. The LLE showed a high amount of background from the matrix, and the sensitivity for the IAC was poor, even when three times the supplier's recommended (5 mL) volume of urine was used. Aliquots of urine greater than 15 mL blocked the columns. Hence, the association of LLE using a 60-mL
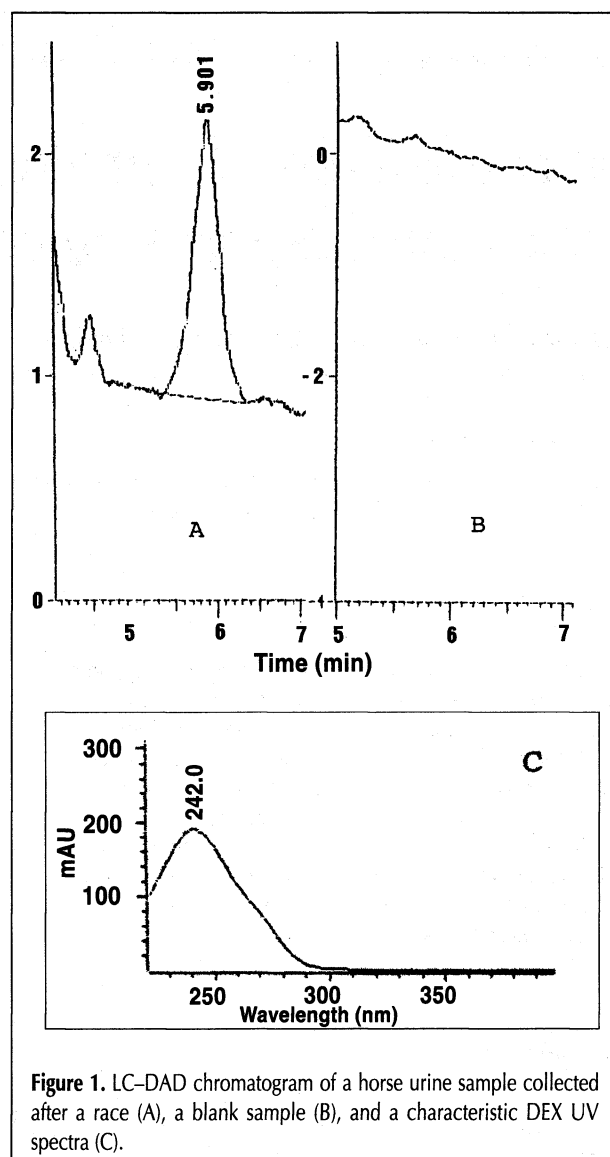

Figure 1. LC–DAD chromatogram of a horse urine sample collected after a race (A), a blank sample (B), and a characteristic DEX UV spectra (C).

aliquot of urine followed by IAC made it possible to obtain the desired sensitivity. The goal of the LLE was to concentrate DEX in the final residue prior to IAC. IAC showed much better selectivity when compared with the ordinary solid-phase extraction columns (5,6) and hence gave highly purified residues, which is a fundamental factor in the analysis of low-concentration drugs in a complex matrix.

The DAD response was found to be linear over the range studied. The calibration curve equation was $y = 0.9744x - 3.7218$. The square of the correlation coefficent (r^2) was 0.9996. The retention time for DEX was 5.9 min. The detection limit of the method was 4 ng/mL, a concentration that showed a match quality greater than 98%.

The concentrations used for the recovery study were chosen based on the binding capacity of the immunoaffinity columns (250 ng for DEX). After the first, fifth, and tenth use of the same column, the recoveries were 56.4, 55.0, and 67.3%, respectively. No attempts were made beyond the supplier's recommendations, but these data show that the column could be used more than 10 times with the same efficiency. No references related to this particular experiment were found in the literature, probably due to

the relatively recent use of these columns. The immunoaffinity column can also be used for the detection of betamethasone and flumethasone. The coefficients of variation for the intraday and interday tests were 10.3 and 14.4%, respectively.

In general, DEX is administered a few hours before a race. Figure 1 shows a chromatogram of a horse urine sample collected after a race.

The proposed method detected DEX up to 28 h after the intra-articular administration, showing that it is suitable for the control of this drug. Quantitation of DEX in urine samples after the administration was not realized because it was not required in drug testing.

Conclusion

The use of immunoaffinity columns for the detection of DEX in racing horse urine samples improved the selectivity of the analysis and gave a clean extract without the need of laborious cleanup procedures. It is also an alternative for those laboratories in which LC–MS is not available.

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